Nonequivalence of Membrane Voltage and Ion-Gradient as Driving Forces for the Bacterial Flagellar Motor at Low Load

Chien-Jung Lo, Mark C. Leake, Teuta Pilizota, and Richard M. Berry
Clarendon Laboratory, Department of Physics, University of Oxford, Oxford, United Kingdom

ABSTRACT Many bacterial species swim using flagella. The flagellar motor couples ion flow across the cytoplasmic membrane to rotation. Ion flow is driven by both a membrane potential \( V_m \) and a transmembrane concentration gradient. To investigate their relation to bacterial flagellar motor function we developed a fluorescence technique to measure \( V_m \) in single cells, using the dye tetramethyl rhodamine methyl ester. We used a convolution model to determine the relationship between fluorescence intensity in images of cells and intracellular dye concentration, and calculated the dye tetramethyl rhodamine methyl ester. We found their relation to bacterial flagellar motor function we developed a fluorescence technique to measure pH concentration. We found 7.0–5.0 and extracellular sodium concentration in the range 1–85 mM. Rotation rates for 0.35-

INTRODUCTION Many species of bacteria swim by rotating their flagella, using ion flux across the membrane to drive the flagellar motor \( (1,2) \). Ions are driven by the ion-motive force that comprises membrane potential \( V_m \) and ion gradient across the membrane \( \Delta p \). In Escherichia coli and Vibrio alginolyticus, motors are powered by proton \( (\mathrm{H}^+) \) and sodium ion \( (\mathrm{Na}^+) \) flux, respectively. Using tethered cells and polystyrene beads attached to flagella to measure the speed of rotation of the E. coli motor, it has been shown that the speed is proportional to protonmotive force \( (\mathrm{PMF}) \) \( (3,4) \) and that under the high load imposed by tethered Streptococcus cells \( V_m \) and the proton gradient \( \Delta p \) are equivalent in driving the motor \( (5,6) \). However, in E. coli, \( V_m \) and \( \Delta p \) are strongly correlated and difficult to manipulate independently. As external pH decreases, internal pH also decreases, but not as much, so that the inward-directed \( \Delta p \) increases. At the same time, the inward-directed \( V_m \) decreases, but not enough to compensate for the increase of \( \Delta p \), with the overall result that the PMF becomes larger \( (7) \). Variation of the speed of the E. coli motor in the pH range 4.7–8.8 \( (8) \) is consistent with the measured PMF variation and proportionality between speed and PMF.

In this work, we studied chimeric sodium-driven flagellar motors in E. coli, containing rotors from the proton-driven E. coli motor and stators that combine proteins from proton-driven E. coli and sodium-driven V. alginolyticus motors \( (9–11) \). To investigate the dependence of the motor mechanism on sodium-motive force \( (\mathrm{SMF}) \), we developed a method to measure \( V_m \) in single cells using the Nernstian fluorescent dye, tetramethyl rhodamine methyl ester (TRM). Combining this method with single-cell measurements of intracellular sodium concentration \( ([\mathrm{Na}^+]_m) \) and motor speed \( (11) \), we demonstrated that \( V_m \) and \( \Delta p \) are equivalent in driving the motor. This confirms earlier, similar, results for the proton-driven motor. However, under low load with 0.35-\( \mu \)m-diameter beads, the speed was greater with high external sodium concentration, indicating that the arrival of sodium ions is the rate-limiting step in low sodium concentration.

Quantitative measurement of \( V_m \) using cationic indicator dyes is based on equilibration of the dye across the membrane according to Boltzmann’s law:

\[
\frac{C_{\text{in}}}{C_{\text{ex}}} = \exp(-qV_m/kT),
\]

where \( k \) is Boltzmann’s constant, \( T \) absolute temperature, \( q \) the charge of the dye, and \( C_{\text{in}} \) and \( C_{\text{ex}} \) the intracellular and extracellular dye concentrations, respectively. For a univalent cation, Eq. 1 can be rearranged to give the Nernst equation

\[
V_m = -2.3 \frac{kT}{q} \log \frac{C_{\text{in}}}{C_{\text{ex}}},
\]

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Address reprint requests to Richard Berry, Clarendon Laboratory, Dept. of Physics, University of Oxford, Parks Rd., Oxford OX1 3PU, U.K. Tel.: 44-0-1865-282559; Fax: 44-0-1865-272400; E-mail: r.berry1@physics.ox.ac.uk.
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The dye should have high membrane permeability to ensure rapid equilibration and low toxicity to avoid perturbing the cell. \( C_{\text{in}} \) and \( C_{\text{ex}} \) are the concentrations of free dye in aqueous solution, as distinct from any dye molecules that are bound to the cell membrane or otherwise immobilized. Thus minimal binding of the dye to membranes and other intra- or extracellular components is desirable, so that the ratio \( C_{\text{in}}/C_{\text{ex}} \) can be determined accurately from fluorescence intensities. A typical bacterial membrane potential is around \(-150 \text{ mV}\), corresponding to \( C_{\text{in}}/C_{\text{ex}} \approx 350\). This can lead to high concentrations of dye inside the cell, which can in turn lead to aggregation and quenching of fluorescence. Whereas this effect can be used as a qualitative indicator of \( V_{\text{m}} \) (12), quantitative measurements should avoid aggregation by using low concentrations of dye. Thus bright fluorescence, low photobleaching, and low aggregation and self-quenching are further desirable properties of the dye. Many hydrophobic potential dye molecules have been designed to determine \( V_{\text{m}} \) of cells and mitochondria in vivo (13–16). In particular, the dye TMRM has been shown to be suitable for quantitative measurements of \( V_{\text{m}} \) in mitochondria (14).

Because bacteria (and mitochondria) are similar in size to the diffraction limit of a light microscope, the fluorescence intensity of pixels within the image of a single bacterium is not simply proportional to \( C_{\text{in}} \), but also includes contributions from adjacent extracellular regions. Typically, \( C_{\text{in}} \) is greater than \( C_{\text{ex}} \), thus the brightness of the cell image is reduced compared to a solution of the same concentration, and the ratio of internal to external fluorescence intensities underestimates the ratio \( C_{\text{in}}/C_{\text{ex}} \). Loew’s group has reported the measurement of \( V_{\text{m}} \) in individual mitochondria using TMRM fluorescence, correcting for the brightness reduction by measuring the point spread function (PSF) of the microscope and modeling the convolution process that reduces the brightness of the mitochondrial image (14,16). In this work, we adapted this technique to develop a single-cell fluorescence measurement of \( V_{\text{m}} \) in bacteria. We imaged \( E. \text{coli} \) cells immobilized at low density on a microscope coverslip, giving a very well-defined geometry well suited to optical convolution modeling. By using a high quantum-efficiency electron-multiplying charge-coupled device (EMCCD) camera, we obtained low-noise images using external TMRM concentrations of 0.1 \( \mu \text{M} \) and image exposure times of 10–30 ms. \( E. \text{coli} \) is a gram-negative bacterium with an outer membrane that acts as a barrier to the permeation of hydrophobic molecules such as TMRM. To increase the permeability of the dye with minimal damage to cell function, we pretreated cells with EDTA (11).

MATERIALS AND METHODS

Bacteria and cultures

\( E. \text{coli} \) strain YS34 (\( \Delta \text{cheY, fliC::Tn10, } \Delta \text{gpiA, } \Delta \text{motAmotB} \)) (10,11,17), with plasmid pYS11(fliC sticky filaments) and a second plasmid for inducible expression of stator proteins were used in our experiments. As described (11), chimeric stator proteins were expressed from plasmid pYS13 \( \text{gomA}p\text{otB7}^{\beta\text{I}} \), induced by isopropyl-\( \beta \)-D-thiogalactopyranoside and wild-type stator proteins were expressed from plasmid pDBB27 \( \text{motA}0\text{motB} \), induced by arabinose. Cells were grown in T-broth (1% tryptone (Difco, Detroit, MI), 0.5 % NaCl) for 5 h at 30° C from frozen stock containing the appropriate antibiotics and inducers (isopropyl-\( \beta \)-D-thiogalactopyranoside, 20 \( \mu \text{M} \), or arabinose, 5 mM).

Sample preparation

Cells were suspended in sodium motility buffer (10 mM potassium phosphate, 85 mM NaCl, pH 7.0) plus 10 mM EDTA for 10 min to increase permeability to dye (11), then washed three times in sodium motility buffer. Cells at a density of \( 10^7 \) cells/ml were suspended in sodium motility buffer plus 0.1 \( \mu \text{M} \) TMRM (MIP, Ann Arbor, MI) and loaded into custom-built flow chambers (volume \( \sim 5 \mu \text{l} \)), in which they were immobilized on polylysine-coated coverslips (11). For speed measurements, cells were sheared to truncate flagella, polystyrene beads (1.0 and 0.35 \( \mu \text{m} \)) were added to reduce aggregation. With low-level induction of stator proteins, speed histograms combining data from many cells under the same conditions showed distinct peaks corresponding to discrete numbers of stator units (see Fig. 4, A and D, and Reid et al. (17)). For 0.35-\( \mu \text{m} \) beads the speed with a single unit was obtained by a Gaussian fit to the slowest peak. For 1-\( \mu \text{m} \) beads the speed/unit was obtained by dividing the speeds with 1–5 units (obtained from a multiple-Gaussian fit to the speed histogram) by the number of units, and then averaging these five independent estimates. Intracellular sodium measurements were performed on single cells using the fluorescent indicator dye Sodium Green as described (11).

Microscopy

Cells were observed in epifluorescence using a custom-built microscope as described (11,19), except that TMRM was excited in epifluorescence mode at 532 nm by a diode-pumped solid-state laser (LCMT111-20, Laser2000, Northants, UK) via a dichroic mirror (530-nm long-pass). The total illuminated area was \( (20 \mu \text{m})^2 \) and the illumination intensity at the sample was \(-5 \text{ W/cm}^2 (\pm 2\% \)\). The sample flow chamber was mounted on a piezo-electric stage (P-517.3c, PI, Karlsruhe, Germany). Fluorescence emission was passed through the dichroic mirror, an emission filter (580-nm long-pass) and a notch rejection filter (532 nm) and imaged at 30 mm/pixel onto a cooled 128 \( \times \) 128-pixel array of a back-illuminated EMCCD camera (iXon, DV860-BI, Andor, Belfast, UK). All experiments were performed at 23° C.

Internal fluorescence intensity

Images of bacteria with pixel intensities \( I(x,y) \) were obtained with the focal plane passing through the center of the bacteria and an exposure time of 10–30 ms. The total fluorescence intensity \( I_F \) of a cell was defined as the average intensity of the central part of the cell, ignoring the marginal area and subtracting the background:

\[
F_T = \langle I - I_{\text{bg}} \rangle_{x>0},
\]

where the background \( I_{\text{bg}} \) and threshold \( I_\text{tr} \) intensities are as defined elsewhere (11). \( F_T \) includes fluorescence due to dye bound to the membrane \( F_m \) as well as fluorescence due to free dye \( F_{\text{free}} \). To estimate \( F_m \) we treated cells, previously loaded for 30 min with TMRM and \( [\text{Na}^+]_o = 85 \mu \text{M} \), with 50 \( \mu \text{M} \) carbonyl cyanide 3-chlorophenylhydrazine (CCCP) for a further
30 min (see Fig. 2 A). CCCP collapses the membrane potential, under which condition we expect \( V_m = 0 \) and \( C_m = C_{ex} \). Any remaining increased fluorescence of the cell compared to the background can be attributed to bound dye. We found that \( V_m \) (and therefore also \( C_m \) and \( F_m \)) depended strongly on pH in but only weakly on [Na⁺]_in, and we estimated, for each value of pH_in studied,

\[
F_m = \langle F \rangle_{cccp},
\]

where the average was taken over ~60 cells after loading and CCCP treatment as described above in buffers of the appropriate pH. The internal fluorescence due to free dye was estimated for each single-cell measurement as

\[
F_m = F_T - F_m.
\]

### External fluorescence intensity

Due to the logarithmic nature of the Nernst equation, \( C_m \) will be ~350 times greater than \( C_{ex} \) when the \( V_m = -150 \) mV. If the camera exposure time were set to match the pixel intensities within the cell image to the full 14-bit dynamic range of the EMCCD, the external fluorescence intensity \( F_{ex} \) would be close to the noise level. Therefore, we obtained \( F_{ex} \) as follows. We filled the flow cell with 0.1 mM TMRM in sodium buffer and imaged empty areas with the focal plane 0.45 \( \mu \)m above the coverslip surface, corresponding to the height of the center of a cell. (The coverslip surface was easily found by searching for slight scratches.) We defined

\[
F_{ex} = \langle I \rangle,
\]

where the average was taken over the entire image. \( F_{ex} \) was measured at a given illumination power with different exposure times in the range 0.1–1.0 s, and \( F_{ex} \) was defined as the slope of a line fit of \( F_{ex} \) versus exposure time, multiplied by the exposure time for cell images, 10–30 ms.

### Point spread function

The PSF, \( P(x, y, z_i) \), was defined as the pixel intensity \( I(x, y) \) in images of single 20-nm fluorescent beads (Molecular Probes, Eugene, OR) stuck to the coverslip at \( x = y = 0 \) and at a distance \( -z_j \) below the focal plane. The piezoelectric stage was used to scan \( z_i \) over the range \( \pm 2.5 \mu \)m in 50-nm intervals (the image pixels are 50 nm², giving a cubic grid). Three images with exposure times of 0.1 s were averaged for each \( z \). The PSF was normalized after background subtraction and correcting for photobleaching (Fig. 1B).

### Optical convolution model

We created a 3D model of a chamber with an E. coli cell stuck to the surface (Fig. 1A). The dye concentrations in the model are

\[
C_i(x, y, z) = \begin{cases} 
0: z \leq 0, \text{glass} \\
C_m: \text{medium}, \\
C_{in}: \text{cell}
\end{cases}
\]

where the subscript \( i \) indicates (50 nm)³ voxels filling the model chamber. The cell is a cylinder (2 \( \mu \)m long, 0.9- \( \mu \)m diameter) capped by two hemispheres (0.9- \( \mu \)m diameter) and stick to the surface. The remaining volume of the chamber, whose \( x, y, \) and \( z \) dimensions are 14 \( \mu \)m, 12 \( \mu \)m, and 3 \( \mu \)m, respectively, is filled with medium. The blurred image, with the focal plane set at a distance \( z_0 = 0.45 \mu \)m from the coverslip, equivalent to midcell height as used for experimental measurements, was calculated as the convolution of the dye distribution and the PSF:

\[
I_m(x, y, z) = \sum_i[C_i(x_i, y_i, z_i)P(x-x_i, y-y_i, z_i-z_0)],
\]

where \( I_m(x, y, z) \) was the modeled intensity of image pixel \( j \) (Fig. 1C, lower). The internal fluorescence intensity \( F_m \) was calculated from the image \( L_m \) as described above for experimental data using Eqs. 3–5, with \( F_m = 0 \), and the external fluorescence intensity \( F_{ex} \) was calculated as the average pixel intensity in a simulated image with \( C_m = C_{ex} = 0.1 \mu \)M. We defined a correction factor \( S(F_{in}/F_{ex}) = (C_m/C_{ex})(F_{in}/F_{ex}) \), where \( C_m \) and \( C_{ex} \) are the concentrations in the model and \( F_m \) and \( F_{ex} \) are the calculated fluorescence intensities (Fig. 1D, inset).

### Calculation of \( V_m \)

We calculated \( V_m \) using the Nernst equation, Eq. 2, and the corrected concentration ratio

\[
C_m/C_{ex} = (F_{in}/F_{ex})S(F_{in}/F_{ex}),
\]

where \( F_{in} \) and \( F_{ex} \) were measured experimentally and the correction factor \( S(F_{in}/F_{ex}) \) was calculated using the optical convolution model.

### RESULTS

#### Dye loading and response time

The bacterial outer membrane is a barrier to hydrophobic molecules such as the membrane potential dye TMRM. We increased the membrane permeability by pretreating cells with EDTA (11). The external TMRM concentration

![FIGURE 1](image-url) Calculation of the correction factor for convolution of the dye distribution with the microscope PSF. (A) Model of the dye distribution for a bacterium attached to a glass coverslip. (B) Cross sections of the measured PSF at different values of \( z \). (C) Dye distribution at midcell height (upper) and the corresponding image after convolution (lower). (D) The calculated fluorescence intensity ratio, \( F_{in}/F_{ex} \), versus the concentration ratio, \( C_m/C_{ex} \). The correction factor, \( S(F_{in}/F_{ex}) = (C_m/C_{ex})(F_{in}/F_{ex}) \), is also shown (inset). Fluorescence intensities were calculated from the convoluted image as described in the text.
$C_{ex} = 0.1 \, \mu M$ was chosen to balance low membrane binding against sufficient fluorescence. After dye loading, we measured flagellar rotation to confirm that the motor and SMF were not affected by the dye. The mean and standard deviation of measured speed of $1-\mu m$ beads attached to the motor were determined from 26 nonloaded cells and 30 loaded cells in 85 mM $[Na^+]_{ex}$, pH 7. For nonloaded cells, the mean speed was $89 \pm 0.6$ Hz and the mean value of the standard deviations in speed for each cell was $1.8 \pm 0.6$ Hz; the corresponding values for loaded cells were $88 \pm 6$ Hz and $1.7 \pm 0.8$ Hz, indicating no significant effect of dye loading.

Fig. 2 A shows the total fluorescence intensity $F_T$ during the loading of dye into live cells in different $pH_{ex}$ (solid symbols). The fluorescence intensities increased exponentially to a steady state within $\sim 30$ min; exponential fits gave time constants of $10 \pm 8$, $14 \pm 2$, and $10 \pm 2$ min (mean $\pm$ SD) for pH 8, pH 7, and pH 6, respectively. The effect of 50 $\mu M$ CCCP, a proton ionophore that eliminates the membrane potential, is shown for cells in pH 7 (open circles). $F_T$ decreases exponentially (within $\sim 30$ min, fitted time constant $12 \pm 3$ min) to a stable nonzero value, $F_T = F_M$, indicating membrane binding of the dye (dashed line).

**CCCP effect**

The effect on cell fluorescence of 40 min incubation with a wide range of CCCP concentrations (0.1–50.0 $\mu M$), at pH 7, is shown in Fig. 2 B (upper). As in Fig. 2 A, the residual fluorescence intensity at high CCCP concentration is due to membrane binding of the dye. We estimated the fluorescence intensity due to membrane binding, $F_m$, as the average fluorescence intensity of cells treated with 50 $\mu M$ CCCP (Methods). Fig. 2 B (lower) shows the membrane voltage, $V_m$, calculated as described in Methods, as a function of CCCP concentration. $V_m$ decreases to zero with increasing [CCCP], indicating that CCCP may be used to achieve low values of $V_m$ in experiments to investigate motor speed or other physiological functions. However, there was considerable intercellular variation in the value of $V_m$ at a given CCCP concentration, as indicated by the large standard deviation (Fig. 2 B, lower, error bars). This illustrates the need to measure $V_m$ in each cell if CCCP is to be used to control $V_m$.

**Dependence of $V_m$ and $\Delta pNa$ upon $pH_{ex}$ and $[Na^+]_{ex}$**

Fig. 3 A shows the dependence of $V_m$ on $pH_{ex}$ for E. coli cells expressing either sodium-driven chimeric or proton-driven wild-type flagellar motors. $V_m$ decreases from $-140 \pm 14$ to $-85 \pm 10$ mV as pH changes from 7 to 5 with chimeric motors, and from $-134 \pm 17$ to $-85 \pm 13$ mV with wild-type motors. We previously showed that sodium influx through the chimeric motors is a significant part of the total sodium flux in a cell (11). The absence of any significant difference in $V_m$ between cells expressing proton- and sodium-driven motors indicates that $V_m$ is not affected by the extra sodium flux through chimeric motors, as expected if $V_m$ is dominated by the proton cycle in E. coli and relatively insensitive to the sodium cycle.

This is further confirmed by the lack of dependence of $V_m$ upon $[Na^+]_{ex}$ (Fig. 3 B). The error bars in Fig. 3, A and B, pH 7 (squares), are standard deviations of measurements of
50 cells and are considerably larger than the estimated single-cell uncertainty in each measurement of $V_m$ (∼3 mV, see Discussion). Thus, the standard deviation reflects intercellular variation in the value of $V_m$ at a given $pH_{ex}$ and $[Na^{+}]_{ex}$. (Other error bars in Fig. 3 represent the mean ± SE of 50 cells measured.)

We previously reported the dependence of the internal sodium concentration $[Na^{+}]_{in}$ and the sodium concentration gradient $\Delta pNa$ upon $[Na^{+}]_{ex}$ in E. coli at pH 7 (11). Here we use the same method to explore further the dependence of $\Delta pNa$ upon $pH_{ex}$ and $[Na^{+}]_{ex}$ (Fig. 3, C and D). $\Delta pNa$ depends weakly upon $pH_{ex}$ (Fig. 3 C) and strongly upon $[Na^{+}]_{ex}$ (Fig. 3 D). We performed least-squares global fits of the dependence of $V_m$ and $\Delta pNa$ upon $pH_{ex}$ and $[Na^{+}]_{ex}$ to a log-linear model:

$$V_m = a + b \times pH_{ex} + c \times \log[Na^{+}]_{ex};$$  

$$\Delta pNa = d + e \times pH_{ex} + f \times \log[Na^{+}]_{ex},$$

where $a$, $b$, $c$, $d$, $e$, and $f$ are fitting parameters and $[Na^{+}]_{ex}$ is in mM. The best-fit parameters for $V_m$ (Fig. 3, A and B) were $a = 57 \pm 4$ mV, $b = -28 \pm 1$ mV/(pH unit), and $c = 0 \pm 1$ mV/decade. For $\Delta pNa$ (Fig. 3, C and D), the best-fit parameters (expressing $\Delta pNa$ in units of mV) were $d = 5 \pm 4$ mV, $e = 5 \pm 1$ mV/(pH unit), and $f = -47 \pm 1$ mV/decade (errors are at 95% confidence limit). The global fits are shown as solid lines in Fig. 3. Thus to a reasonable approximation, $V_m$ depends upon $pH_{ex}$ but not upon $[Na^{+}]_{ex}$, and $\Delta pNa$ depends upon $[Na^{+}]_{ex}$ but not upon $pH_{ex}$. This allows independent control of the two components of the SMF using pH and sodium concentration as the control parameters.

**Motor speed versus SMF**

First we measured the speed of 1-µm polystyrene beads attached to chimeric flagellar motors (11,17) as a function of $pH_{ex}$ and $[Na^{+}]_{ex}$ (Fig. 4, A–C). Fig. 4 C shows the data of Fig. 4 B as speed versus SMF. The motor speed varies linearly with SMF, from 8.7 to 2.2 Hz/stator over the range $-187$ to $-54$ mV. In this high load condition, the two components of SMF, $V_m$ and $\Delta pNa$, are equivalent to within the limits of experimental uncertainty. Similar measurements, using 0.35-µm beads, are shown in Fig. 4, D–F. For a given value of $[Na^{+}]_{ex}$ (and thus also $[Na^{+}]_{in}$ and $\Delta pNa$), the motor speed varies linearly with SMF as $V_m$ is varied via changes in $pH_{ex}$. However, the slope, and therefore the motor speed at a given SMF, is lower at low $[Na^{+}]_{ex}$ than at higher $[Na^{+}]_{ex}$. $V_m$ and $\Delta pNa$ are not equivalent as driving forces of the flagellar motor in the low-load region.

**DISCUSSION**

**Accuracy and error estimation**

The sources of error in our single-cell fluorescence $V_m$ measurement are as follows:

1. Random error in measurements of fluorescence intensities $F_{in}$: the standard deviation of successive measurements of the same cell was typically 3%, attributed to instrumental noise.
2. Focusing error: focusing on the mid-cell plane was judged by the user, introducing a possible source of uncertainty. By repeating the focusing process on the same cell 10 times and reading the z-positions from the
piezoelectrical stage driver, we determined a standard deviation of 60 nm in the focusing height. This contributes 2% uncertainty to fluorescence intensity measurements and a further 2% to the value of the correction factor $S(F_{\text{in}}/F_{\text{ex}})$.

3. Cell-size effect on the correction factor: the diameter of *E. coli* is $0.8–1.0 \mu m$ and the length is $3 \mu m$. By varying the dimensions of model cells in the optical convolution calculation, we found that variations of cell length had negligible effect on the correction factor, whereas the uncertainty in cell diameter leads to an uncertainty of 11% in the correction factor.

4. Measurement and fitting error in $F_{\text{ex}}$: the fitting error is typically 1%.

5. Membrane binding fluorescence intensity $F_{\text{m}}$: the fluorescence intensity measurements from cells after CCCP treatment showed a standard deviation of 22%. The relatively high variation may be due to intercellular variation of $V_m$. The measured standard deviation in $V_m$ of $\sim 15 \text{ mV}$ corresponds to a fivefold variation in dye concentration inside the cell; the higher the dye concentration inside the cell, the higher the expected level of membrane binding.

Combining the contributions of these uncertainties to our estimate of $V_m$ (Methods), the uncertainty is $<3\%$ at negative values beyond $-110 \text{ mV}$, increasing to 5% at $-90 \text{ mV}$, 10% at $-70 \text{ mV}$, 50% at $-40 \text{ mV}$, 100% at $-30 \text{ mV}$. Precision at high membrane voltages is helped by the logarithmic nature of the Nernst equation. Under our experimental conditions, $V_m$ in *E. coli* cells had negative values $>-80 \text{ mV}$ and the uncertainty is $< -6\%$. To increase the accuracy of measurements of low values of $V_m$, we can calibrate the membrane binding of each individual cell by applying 50 $\mu \text{M CCCP}$ at the end of an experiment, removing this contribution to the uncertainty. By this method, the estimated uncertainty is $< 5\%$ at $-80 \text{ mV}$, 18% at $-40 \text{ mV}$, and 100% at $-10 \text{ mV}$.

![FIGURE 4 The speed of chimeric flagellar motors. (A) A histogram of all speeds recorded from 50 different motors driving 1-\mu m diameter beads at pH 7 and [Na$^+$]$_{\text{ex}}$ = 85 mM. (B) Average speed/stator unit with 1-\mu m beads versus pH$_{\text{ex}}$ for different [Na$^+$]$_{\text{ex}}$. (C) Speed versus SMF for the data in B. (D) Histogram of speeds from 25 motors driving 0.35-\mu m diameter beads at pH 7 and [Na$^+$]$_{\text{ex}}$ = 85 mM. (E) Average speed of the first stator unit with 0.35-\mu m beads, versus pH$_{\text{ex}}$ for different [Na$^+$]$_{\text{ex}}$. (F) Speed versus SMF for the data in E. Also shown in C and F are linear fits constrained to the origin for data at 1 mM (dashed lines), 10 mM (dotted lines), and 85 mM (solid lines) [Na$^+$]$_{\text{ex}}$. Fitted gradients for 1 mM, 10 mM, and 85 mM [Na$^+$]$_{\text{ex}}$, respectively, are $-0.045 \pm 0.001 \text{ Hz/stator/mV}$, $-0.045 \pm 0.002 \text{ Hz/stator/mV}$ and $-0.046 \pm 0.001 \text{ Hz/stator/mV}$ in C, and $-0.23 \pm 0.01 \text{ Hz/stator/mV}$, $-0.32 \pm 0.003 \text{ Hz/stator/mV}$, and $-0.36 \pm 0.005 \text{ Hz/stator/mV}$ in F.](image-url)
Comparison to other $V_m$ measurements

Our measurements of $V_m$ in single *E. coli* cells are consistent with previous results. Felle et al. measured $V_m$ in *E. coli* using the distribution of radio-labeled permeant cations TPP$^+$ and TPMP$^+$ (20). The distribution of these ions follows the Nernst equation and their uptake can be determined by dialysis or filtration. They found that $V_m$ increased linearly from $-80$ mV at pH 5 to $-130$ mV at pH 7, a slope of $-25$ mV/pH unit (compared to our measured value of $-28$ mV/pH unit). Also using *E. coli* and the same experimental method, Castle et al. measured $V_m = -176$ mV at pH 8.5 and $-130$ mV at pH 6.5 ($-23$ mV/pH unit) (21), and Minamino et al. (7) measured $V_m = -130$ mV at pH 8.0 and $-54$ mV at pH 5.0 ($-24$ mV/pH). Novo et al. measured $V_m = -120$ mV in *Staphylococcus aureus* and *Micrococcus luteus* using the aggregation and redshift of cyanine dye (22). Suzuki et al. measured $V_m$ changes induced by a K$^+$ diffusion potential in *E. coli* and *Rhodospirillum rubrum* using the aggregation and quenching of carbocyanine dye (23). This cationic dye can permeate the membrane freely and redistribute according to the Nernst equation and thus quenches the fluorescence of the whole sample. However, the authors did not report measurement of the natural $V_m$ in either species.

Unlike our method, all of the above measurements represent ensemble averages of a large number of cells. Felle et al. measured $V_m = -135$ mV at pH 7 in giant *E. coli* spheroplasts using a patch-clamp micropipette. Although this is a single-cell method, the production of giant spheroplasts is more disruptive than our method and in particular requires disruption of the cell wall, which makes simultaneous measurements of the flagellar motor impossible. Lowe et al. measured $V_m \approx -150$ mV in individual mitochondria in neuroblastoma cells using a method very similar to ours (14). Our data show large intercellular variation, $\pm 15\%$, in the measured values of $V_m$ at a given pH. We measured similar cell-to-cell variation in $\Delta pNa$ at a given $[\text{Na}^+]_{ex}$ (11), indicating that individual variation between genetically identical cells extends to both components of the SMF. Based on extrapolation of the linear relationship between motor speed and $\Delta pNa$, we previously predicted that $V_m$ would be $-137$ mV at pH 7, in close agreement with our measured value of $-140 \pm 2$ mV (mean $\pm$ SE) in this work. We found that $\sim 30$ min was required for equilibration of dye concentrations in our experiments, presumably due to relatively low permeability of the outer membrane to dye, after our treatment with EDTA. This limits the time resolution of our technique. Faster response times may be possible by altering our method of permeabilizing the outer membrane, but care would be needed to quantify damage to cells that may be caused by such treatments.

Instability at alkaline pH$_{ex}$

The pH range 5–7 (Figs. 3 and 4) was chosen because motor rotation was stable over this range. Many chimera motors were unstable at pH$_{ex} = 8$ and sometimes stopped after a brief period of rapid rotation. $V_m$ measurement in cells at pH$_{ex} = 8$ indicated that this was due to a collapse of $V_m$ (see Supplementary Material).

Sodium energetics in *E. coli*

We have shown that $V_m$ in *E. coli* varies linearly with pH$_{ex}$ and is independent of $[\text{Na}^+]_{ex}$ and of the sodium flux through chimeric flagellar motors. This indicates that the membrane voltage is dominated by the balance of proton fluxes, and varies in such a way as to stabilize the PMF and internal pH as pH$_{ex}$ changes (7, 21). $\Delta pNa$, on the other hand, depends strongly on $[\text{Na}^+]_{ex}$ and only weakly on pH$_{ex}$, allowing independent control of the two components of the SMF over the ranges $-85$ to $-140$ mV in $V_m$ and $-60$ to $+40$ mV in $\Delta pNa$. Larger values of $V_m$ are possible using alkaline pH, up to a maximum observed in this work of $V_m = -165$ mV and SMF $= -210$ mV, but the SMF is not stable under these extreme conditions in cells expressing Na$^+$-driven chimeric flagellar motors. There is no strong evidence for the existence of a primary sodium pump in *E. coli*, and the best candidate for maintaining the SMF is the sodium/proton antiporter (24). This is consistent with the weak linkage we observed between the SMF and proton energetics, especially if both components of the PMF are equally effective in maintaining the SMF.

Motor function and SMF or PMF

The relationship between motor speed and ion-motive force has been reported in different bacteria with different methods. Note that swimming speed does not directly reflect motor function in *E. coli* because swimming depends upon the cooperation of flagella in bundles, and therefore early experiments with swimming *E. coli* cells are difficult to interpret. Berg’s group showed a linear relationship between motor speed and PMF up to $-85$ mV in tethered *Streptococcus* cells, using a K$^+$ diffusion potential and a pH gradient to drive motors on starved cells (5, 6). Further studies on glycolyzing tethered *Streptococcus* cells have suggested that the linear relationship between speed and PMF extends up to $-150$ mV (25). Manson et al. also showed that the pH gradient and $V_m$ were equivalent in tethered *Streptococcus* cells (5). Using a micropipette to energize filamentous *E. coli* cells, with beads acting as markers attached to flagellar motors, Fung and Berg demonstrated the proportionality between speed and $V_m$ up to $-150$ mV (3). By observing a cell tethered to the coverslip by one motor (operating under high load) and measuring simultaneously the rotation of a second motor on the same cell (operating under lower load, marked by a 0.4-μm-diameter bead), Gabel and Berg showed that the speeds of the two motors were proportional when the PMF was gradually eliminated by adding the ionophore CCCP (4). The high-load motor in this experiment acted as an indicator of the PMF of the cell, as previous work had shown.
that speed is proportional to PMF under high load. Thus, the experiment demonstrated that speed varies linearly with PMF under both load conditions, although the relative contributions of $V_m$ and pH gradient were not known. The motor speed of sodium-driven motors in Vibrio alginolyticus has a linear dependence upon $\log[[\text{Na}^+]_{\text{ex}}]$, but the SMF was not measured (26).

In this work, we manipulated both components of the SMF by changing the pH and sodium concentration of the external medium. Sodium-driven chimeric flagellar motors under high loads imposed by 1-μm beads rotated stably at $pH_{\text{ex}}$ between 5 and 7, and speed varied linearly with SMF from $-53$ to $-187$ mV, with no detectable difference between the two components of the SMF (Fig. 4 C). The two components of SMF were measured separately, with high accuracy, in single cells, and could be controlled on a timescale of $\approx 5$ s by rapid exchange of the medium. We did not measure $V_m$ and $\Delta pNa$ simultaneously in the same cell due to overlap in the emission spectra of the indicator dyes used for each measurement. Simultaneous measurements would be possible if a different dye were used for one of the measurements. The linear dependence of speed on SMF in the high-load regime is consistent with tight coupling and high efficiency (27); the motor speed is limited neither by internal conformational changes nor by the movement of ions at these speeds. However, in the low-load regime, the motor speed shows different dependence on $V_m$ and $\Delta pNa$ (Fig. 4 F). Motor speeds in low $[\text{Na}^+]_{\text{ex}}$ are slower than those in high $[\text{Na}^+]_{\text{ex}}$ for a given SMF. All previous comparisons of membrane voltage and ion gradient as driving forces for the flagellar motor have been under high load and have found that the two are equivalent, consistent with high efficiency and near-equilibrium behavior. At high speed and low load, we are observing the rates of reactions in the motor cycle far from equilibrium, and under these conditions the ion gradient and membrane voltage are not equivalent. The simplest explanation is that the diffusion-limited binding of sodium ions is the rate-limiting step at low load in low $[\text{Na}^+]_{\text{ex}}$. By controlling the SMF via $V_m$ and $\Delta pNa$, it is possible to drive the motor purely by $V_m$, setting $\Delta pNa$ at 0. It may also be possible to drive the motor purely by $\Delta pNa$, using CCCP to collapse $V_m$ and transient changes in $[\text{Na}^+]_{\text{ex}}$ to generate a sodium gradient. Further experiments under various loads are needed for a better understanding of the motor. In particular, it will be interesting to measure torque-speed relationships with different magnitudes and components of the SMF and to use these to test models of the motor mechanism that distinguish between the contributions of electrical and chemical potential.

Driving a 1-μm bead, the motor works in the high-load regime close to thermodynamic equilibrium and the efficiency is close to 100%. For cells in a medium of pH 7 and $[\text{Na}^+]_{\text{ex}} = 85$ mM, the motor speed is $8.6 \pm 0.8$ Hz/stator and the SMF is $-187 \pm 15$ mV. The drag coefficient is $20 \pm 2$ pN nm Hz$^{-1}$ and the work done against viscous drag in one revolution is $1087 \pm 144 \times 10^{-21}$ J/stator. By energy balance, the minimum number of ions needed for one revolution is $36 \pm 6$, given by the work done divided by the free energy/ion ($-e \times \text{SMF} = 30 \pm 2 \times 10^{-21}$ J). With the motor driving $0.35$-μm beads at low SMF ($[\text{Na}^+]_{\text{ex}} = 1$ mM and pH 5), a minimum of $20 \pm 9$ ions are required for one revolution, based on a similar calculation. Direct observation of 26 steps/revolution in the chimeric flagellar motor at low load and low SMF (10) indicates that the number of ions required for one step at high load is $>1$, unless the step size is smaller at high load. At low load, it is likely that the efficiency drops and that the actual number of ions/revolution is greater than the minimum required by energy balance. Sowa et al. (10) observed steps under conditions where motor rotation was unstable. In this work, we have identified conditions for stable slow rotation, which will allow investigation of the statistical properties of motor stepping.

CONCLUSION

Combining single-cell $V_m$ and intracellular sodium measurements, the SMF of cells can be determined. In the pH range 5.0–7.0 and external sodium concentration 1–85 mM, the SMF varies from $-53$ to $-187$ mV. The motor speed is stable and varies from 2.2 to 8.7 Hz/stator for 1-μm beads, and from 12.1 to 71.0 Hz for a single stator and 0.35-μm beads. The motor speed when driving 1-μm beads varies linearly with SMF, consistent with tight coupling between ion flux and rotation. The motor speed when driving 0.35-μm beads shows nonequivalent contributions of membrane potential and sodium gradient. Further experiments with different loads are needed to build up a complete motor model. Integration of the fluorescence methods we have described will yield a valuable tool for understanding the energetics and mechanism of the flagellar motor.

SUPPLEMENARY MATERIAL

An online supplement to this article can be found by visiting BJ Online at http://www.biophysj.org.

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